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Structural and immunochemical identification of Le^b glycolipids in the plasma of a group O Le(a-b-) secretor

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Total non-acid glycosphingolipids were isolated from the plasma of a healthy red blood cell group O Le(a-b-) salivary ABH secretor individual. Glycolipids were fractionated by HPLC and combined into eight fractions based on chromatographic and immunoreactive properties. These glycolipid fractions were analysed by thin-layer chromatography and tested for Lewis activity with antibodies reactive to the type 1 precursor (Le^c), H type 1 (Le^d), Le^a and Le^b epitopes. Fractions were structurally characterized by mass spectrometry (EI-MS and LSIMS) and proton NMR spectroscopy. Expected blood group glycolipids, such as H type 1, (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1Cer) were immunochemically and structurally identified. Inconsistent with the red cell phenotype and for the first time, small quantities of Le^b blood group glycolipids (Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glcβ1-1Cer) were immunochemically and structurally identified in the plasma of a Lewis-negative individual. These findings confirm recent immunological evidence suggesting the production of small amounts of Lewis antigens by Lewis negative individuals.

Keywords: Lewis negative, Lewis antigens, secretor, plasma, H type 1, mass spectrometry, nuclear magnetic resonance spectroscopy

Abbreviations: HPLC, high performance liquid chromatography; TLC, (high performance) thin layer chromatography; EI-MS, electron impact ionisation mass spectrometry; LSIMS, liquid secondary ion mass spectrometry; NMR, nuclear magnetic resonance spectroscopy. The sugar types are abbreviated to Hex for hexose, HexNAc for *N*-acetylhexosamine and dHex for deoxyhexose (fucose). The ceramide types are abbreviated to d for dihydroxy and t for trihydroxy base, n for non-hydroxy and h for hydroxy fatty acids; LCB, long chain base.

Introduction

The Lewis histo-blood group system is comprised of two major antigens, Le^a and Le^b, which are synthesized by exocrine epithelial cells, mostly of endodermal origin. These antigens are shed into the exocrine secretions and plasma, and as plasma glycolipids are acquired by cells of the peripheral circulation (reviewed in [1]). The expression of Lewis antigens is the result of epistatic interaction of the products of the genetically independent Lewis and Secretor loci. The Lewis genotype determines if an individual will express Lewis antigens, while the secretor genotype determines what type of Lewis antigen will be expressed and whether soluble ABH antigens will be found in secretions (reviewed in [1]). Although the Lewis-negative phenotype Le(a-b-) is easily recognized, immunological expression of Lewis antigens of different tissues and bodily fluids of these individuals has been reported [2–9]. In a study of plasma glycolipids of Lewis-negative individuals it was found that some Lewis-negative non-secretors have 5-sugar glycolipids reactive with anti-Le^a, and all Lewis-negative secretors have 6-sugar glycolipids reactive with anti-Le^b [3]. Although it is assumed that these antigens represent the appropriate Lewis structures, their unambiguous identification requires structural analysis.

In order to unambiguously identify the Lewis reactive glycolipids in a Lewis-negative secretor-positive individual, total plasma non-acid glycolipids were HPLC fractionated and each fraction was immunologically characterized and then structurally analysed by EI-MS, LSIMS and NMR.

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Materials and methods

Glycolipids

Twelve hundred ml of plasma was obtained by plasmapheresis on two occasions from a healthy blood donor. The blood group, salivary ABH typing and immunochemistry of the total plasma glycolipids of this individual (no. 121 in [3]) and others of the same and related phenotypes have previously been reported. Total non-acid glycosphingolipids were prepared as previously described [10] and fractionated by HPLC (LKB, Bromma, Sweden) on a 10 μm silica column (Maxsil 10 silica, model OOH-0060-PO, Phenomenex, CA, USA) using a chloroform:methanol:water gradient (80:20:1 to 40:40:12 by volume). The separations were monitored by TLC and immunostaining, then combined into eight fractions with similar chromatographic and immunostaining properties.

Thin-layer chromatography and immunostaining

TLC glass plates (Whatman Ltd, England) loaded with about 20 mg of the total fraction and 1 mg of purified fraction were chromatographed in a solvent system of chloroform: methanol:water, ratio 60:35:8 by volume, dried and re-chromatographed in fresh solvent. Chemical detection was done with the anisaldehyde reagent [10], and immunostaining was performed by a modification of the method of Magnani [11, 12]. Monoclonal antibodies used in the immunostaining technique were; anti-Lea 069 (clone BRIC 87) from South West Regional Transfusion Centre, Southmead, Bristol, UK; anti-Leb 073 (clone LM129/181 – note this reagent is an anti-Leab reagent against isolated glycolipids) and anti-LebH 075 (clone LM137/276) from Glasgow and West of Scotland Blood Transfusion Service, Law Hospital, Carluke, UK. The immunological and serological properties of these reagents have been described in detail elsewhere [3, 13]. Affinity purified polyclonal rabbit anti-Lec (lacto-N-biose) was prepared as described [14], by hyper-immunizing a rabbit with boiled saliva from a Le(a-b-) nonsecretor individual and then affinity purifying the specific antibodies of the hyperimmune serum on Synsorb' synthetic Lec (lacto-N-biose) disaccharides (Galb1-3GlcNAc) (Chembiomed, Alberta Research Council, Edmonton, Canada). The immunological properties of this antiserum with glycolipids has been described elsewhere [3]. The nomenclature used for the blood group glycolipids is the epitope name, and when required or known it is followed by the number of sugar units and then the chain type (if any variations exist) e.g. H-5-1 is a 5 sugar H structure based on a type 1 chain, whereas Lea-5 is a 5 sugar Lea structure.

Mass spectrometry

EI-MS of permethylated [15, 16] and permethylated-reduced glycolipid derivatives [17, 18] was made at 70 eV. LSIMS of permethylated-reduced samples was carried out using a matrix of 3-nitrobenzyl alcohol:glycerol 1:1 with 0.1% trifluoroacetic acid and a 30 kV Cs-gun source. Mass spectrometry was done with a double focus trisector mass spectrometer (Autospec, VG Analytical, Fisons Instruments, Manchester, UK). Mass-to-charge values quoted in the text and in Figs 4 and 6 are experimental while the values in Fig. 5 are calculated.

Nuclear magnetic resonance spectroscopy

Native glycolipid fractions were deuterium exchanged in excess $CHCl_3$:CD_3OD (2:1 by volume) and then dissolved in 0.5 ml d6-DMSO:D_2O (98:2 by volume) for proton NMR analysis at 60°C [19]. Permethylated-reduced glycolipid samples were dissolved in 0.4 ml $CDCl_3$ and proton NMR spectra were recorded at 40°C [20]. Spectra were recorded either at 14.1 T (600 MHz) on a Varian Unity 600 (Varian, Palo Alto, CA, USA) with a digital resolution of 0.16 Hz per point, or at 11.7 T (500 MHz) on a JEOL Alpha 500 (JEOL, Tokyo, Japan) with a digital resolution better than 0.5 Hz per point. Chemical shifts are given relative to tetra methyl silane (TMS), and sometimes using the internal solvent peak. Processing was done off-line using the NMR1 program (New Methods Research Inc., Syracuse, NY, USA). Resolution enhancement was achieved using either Lorentz-to-Gauss transformation or Maximum Entropy calculations as implemented in NMR1. Two-dimensional data were recorded using standard pulse sequences provided with the instrument.

Results

From 1200 ml of Le(a-b-) secretor plasma 13.8 mg of non-acid glycolipids were isolated. After fractionation and pooling, eight fractions with similar TLC and immunostaining properties were obtained (Table 1). The first three fractions, representing the mono-, di- and triglycosylceramides, accounted for about three quarters of the total weight (10.1 mg), contained no H or Lewis blood group activity, and were not further analysed. Of the remaining fractions, 4–7 were suitable for TLC, MS and NMR analysis while there was insufficient material in fraction 8 for full structural analysis. Shown in Fig. 1 are the structural formulae for glycolipids discussed in this paper.

Fraction 4 glycolipids migrated by TLC as tetraglycosylceramides and were unreactive with the antisera used (Fig. 2). EI-MS, LSIMS and NMR analyses showed that this fraction contained predominantly globotetraosylceramide (globoside) with traces (< 2%) of globotriaosylceramide and paragloboside. There was no immunological or structural evidence for lactotetraosylceramide.

Fraction 5 glycolipids migrated by TLC as tetra- and pentaglycosylceramides (Fig. 2). This fraction was unreactive with the anti-Le^c, -Le^a and -Le^b reagents but was reactive with anti-Le^{bH} in the 5-sugar region, suggesting the presence of H-5-1 glycolipids. After EI-MS, LSIMS and NMR analyses this fraction was found to contain predominantly paragloboside and H-5-1 (ratio 1.5:1). Traces of globoside and another unidentified fucosylated pentaglycoslyceramide were also

Table 1. Non-acid glycolipid fractions isolated from the plasma of a Le(a-b-) secretor individual, corresponding with lanes as seen on the thin-layer plates (Fig. 2). Immunoreactivity and identities of glycolipids found in each fraction based on thin-layer chromato-graphy, immunostaining, EI-MS, LSIMS and NMR (where possible) are summarized. Different ceramide species found are not shown.

		Immunoreactivity				
Fraction	*mg*	*–Le^{c}*	*–Le^{a}*	*–Le^{b}*	*–Le^{bH}*	*Glycolipids identified**
4	2.1	–	–	–	–	Globoside Globotriaosylceramide Paragloboside
5	0.6	–	–	–	+	Paragloboside H-5-1 Globoside (Le^{x}-5 and/or Le^{a}-5)
6	0.8	–	+	–	+	H-5-1 Le^{x}-5 (and Le^{a}-5) Globopentaosylceramide
7	0.1	+	+	+	+	Le^{b}-6 (Le^{x}-5 and Le^{y}-6) (Globopentaosylceramide) (Linear hexaglycosylceramide) (Fucosylated heptaglycosylceramide)
8	0.1	+	+	+w	–	(Fucosylated heptaglycosylceramide) (Branched fucosylated nonaglycosylceramide) (Linear difucosylated octaglycosylceramide)
T (Total)	13.8	+	–	+w	+	

*Glycolipids indicated in parentheses are only tentatively identified.

Structure	Name
Galα1-4Galβ1-4Glcβ1-1Cer	globotriaosylceramide
GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	globotetraosylceramide or globoside
Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	lactotetraosylceramide
Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	neolactotetraosylceramide or paragloboside
Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	H type 1 or H-5-1
Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	Le^{a}-5
Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	Le^{X}-5
Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	globopentaosylceramide
Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	Le^{b}-6
Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	Le^{Y}-6

Figure 1. Structural formulae for selected blood group antigens and their alternative names as discussed in this paper.

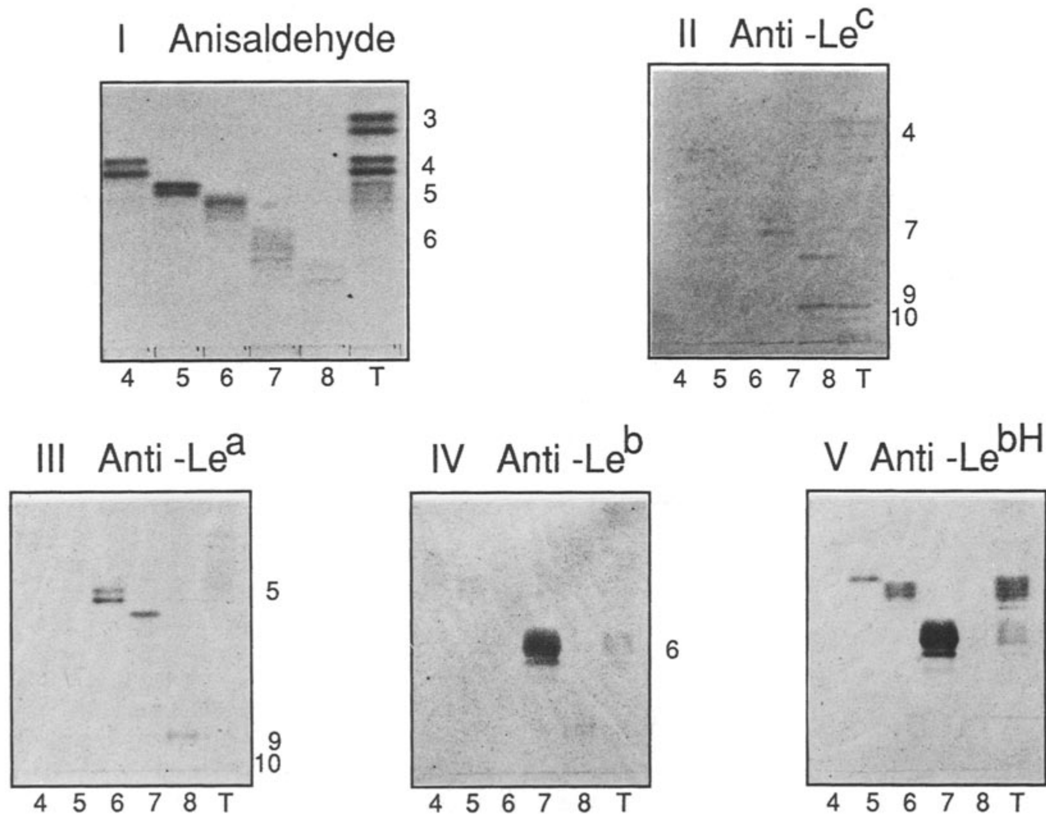

Figure 2. Thin-layer chromatographic analysis of non-acid glycolipid fractions isolated from plasma of a blood group O Le(a-b-) secretor individual. Lanes labelled 4 – 8 indicate the HPLC fractions of the total sample (Lane T) as described in Table 1. The numbers on the right hand side of the chromatograms indicate the approximate number of sugar residues in each glycolipid band. The antibodies used for immunostaining were; plate II= affinity purified rabbit polyclonal anti-Le^c, plate III = 069 anti-Le^a, plate IV = 073 anti-Le^b, plate V = 075 anti-Le^{bH}.

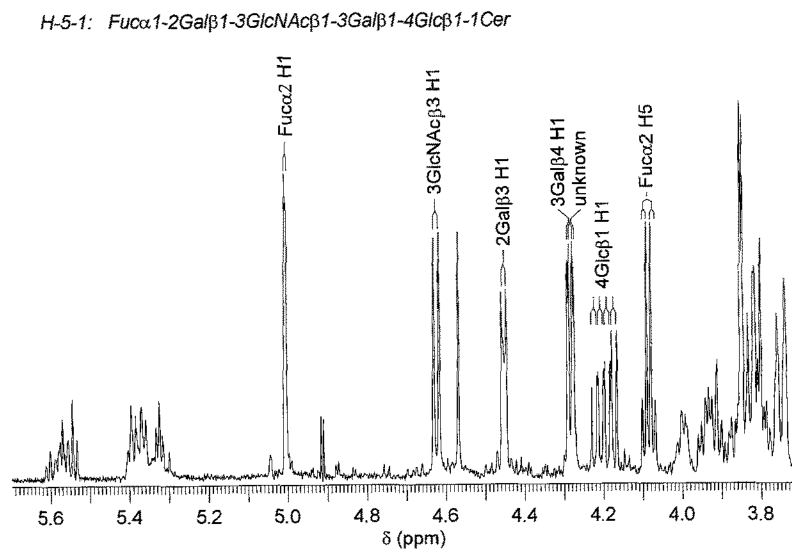

Figure 3. Anomeric region of the 600 MHz proton NMR spectrum, recorded at 60°C in DMSO:D_2O, of native fraction 6 glycolipids. Apart from the indicated H-5 type 1 signals and the unidentified doublet discussed in the text, impurities are seen at 4.56 ppm and 4.91 ppm. The carbohydrate sequence of H-5 type 1 is shown on top.

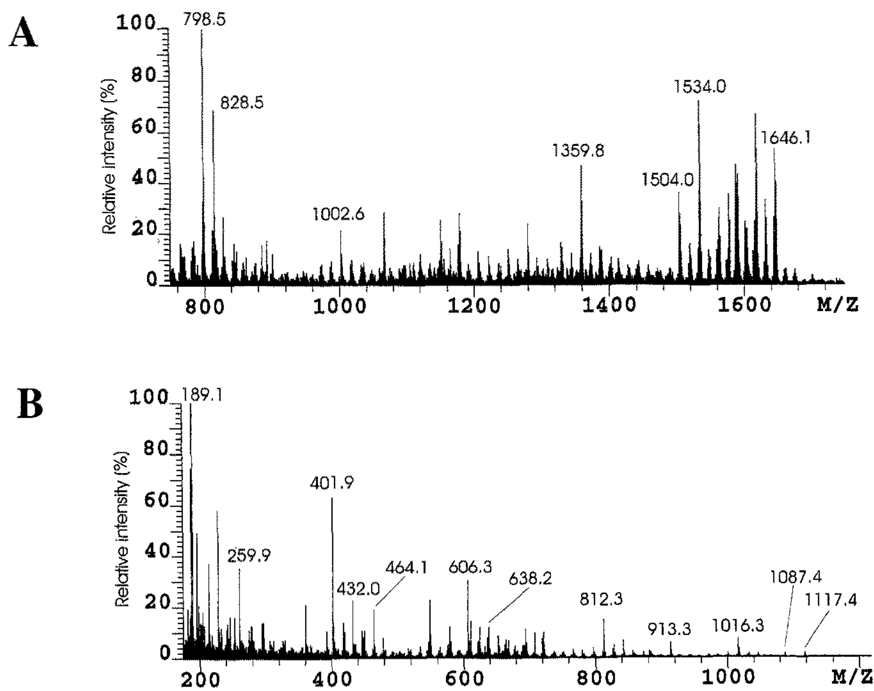

Figure 4. EI-MS spectra of permethylated-reduced (A) and permethylated (B) glycolipids showing characteristic ions for a difucosylated hexaglycosylceramide (Le^b) as isolated in fraction 7.

unidentified fucosylated pentaglycoslyceramide were also present. There were no NMR signals corresponding to H-5-2.

Fraction 6 glycolipids migrated by TLC as pentaglycosylceramides. This fraction was reactive with anti-Le^a and anti-Le^bH but was unreactive with anti-Le^b (Fig. 2), suggesting the presence of Le^a-5 and H-5-1 glycolipids. EI-MS of this fraction revealed immonium and sequence ions diagnostic for a fucosylated pentaglycosylceramide with a variety of fatty acyl chains from n16:0 to h24:0 (not shown). Sequence ions for a type 1 chain were also present [21].

Analysis of this fraction by 600 MHz NMR spectroscopy (Fig. 3) revealed a signal at 5.006 ppm assigned to the α-anomeric proton, a quartet at 4.087 ppm assigned to the C5 proton, and a doublet at 1.065 ppm assigned to the methyl protons of an α1,2 linked fucose. Two doublets, having large spin-spin couplings, at 4.624 ppm and 4.453 ppm can be assigned to β-anomeric protons and together with a singlet at 1.834 ppm, assigned to an *N*-acetyl group, they indicate the presence of a fucosylated Galβ1-3GlcNAcβ trisaccharide in the glycolipid. This identification is confirmed by the cross-peak patterns for the 5.006, 4.624 and 4.453 ppm signals in a 2D-TOCSY spectrum (data not shown). In the region where internal monosubstituted β-galactose and β-glucose signals appear (4.30–4.15 ppm) we had 12 signals (i.e. six doublets). Of the two β doublets around 4.284 ppm (with a total relative integral of 1.4), the low field signal (4.286 ppm) was the result of a β1,4 linked galactose, whereas the high field signal (4.282 ppm) most likely belonged to a smaller molecule. These signals coincided at 500 MHz. The possibility that this split signal was due to ceramide interactions, seen only at 600 MHz was considered. Although this could not be entirely eliminated, 500 MHz 2D correlated spectroscopy (COSY) suggests the presence of two components (results not shown). The relaxation properties, line width and nuclear Overhauser enhancements show a size difference between these two molecules. The other four doublets (with a relative integral of 1.3) at 4.224, 4.208, 4.191, and 4.174 ppm can all be assigned to a β-glucose. This is also evident from the 2D correlation experiments. From these data the structure can conclusively be identified as H-5-1. The chemical shifts of the three outermost sugars corresponded almost perfectly with data from reference spectra run at Göteborg University, but were slightly different from literature data (e.g. [22]). The complex ceramide composition (sphingosine, phytosphingosine with hydroxy and non-hydroxy fatty acids) gives rise to four different anomeric glucose signals, as has been noted before [19]. The different ceramide composition for H-5-1 of fraction 5 (not shown) did not give rise to this splitting of lines (and was otherwise identical to fraction 6).

It was also clear that minor components are present in this fraction. There were potentially three fucose methyl signals at approximately the 5% level, and also the *N*-acetyl region showed three potential *N*-acetamidohexoses at this level or lower. In the anomeric region one could find two sets of anomeric signals corresponding to globopentaosylceramide and the Le^x structure, respectively. Of course only the terminal saccharides are identifiable, since their lactosyl units are more or less identical to the H-5-1 lactosyl unit. A terminal Le^x

galactose would also be overlapping some lactosyl anomeric signals [23]. There were no NMR signals corresponding to H-5-2.

The data when taken together was consistent with fraction 6 containing predominantly H-5-1 and traces of Le^{x} and globopentaosylceramide. Despite an immunological reaction with the anti-Le^{a} reagent no Le^{a}-5 was seen in the NMR spectrum. It is probable that this immunological reaction is due to crossreactivity of this reagent with Le^{x} [13], or alternatively, to the presence of very low levels of Le^{a}-5.

Fraction 7 glycolipids migrated by TLC as penta- and hexaglycosylceramides. This fraction was reactive with anti-Le^{a} in the 5-sugar region and anti-Le^{b} and anti-Le^{bH} (Fig. 2) in the 6-sugar region, suggesting the presence of Le^{a}-5 and Le^{b}-6 glycolipids. The lack of reactivity of the anti-Le^{bH} reagent in the 5-sugar region suggested an absence of H-5-1. Weak reactivity with anti-Le^{c} in the 6–7 sugar region suggested the presence of an extended precursor.

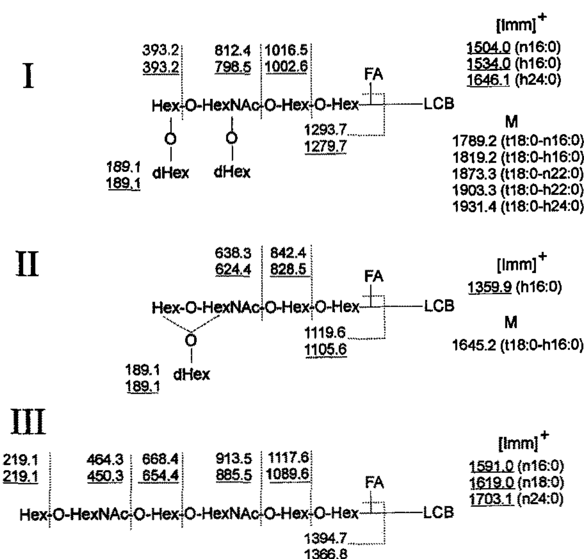

Figure 5. Calculated monoisotopic ^{12}C mass values for molecular and fragmentation ions of methylated and methylated-reduced (underlined) blood group glycolipids indicated in this paper. In drawing II the dHex may be linked in either of two positions, as indicated by the broken line.

EI-MS of the permethylated-reduced fraction produced immonium ions (Fig. 4A) for a difucosyl-hexaglycosylceramide (Fig. 5:I), with various fatty acyl chains at *m/z* 1504.0, 1534.0, and 1646.1. Oxonium ions consistent with a difucosylated hexasaccharide were seen at *m/z* 189.1, 798.5 and 1002.6. Immonium ions consistent with a hydroxy 16:0 fucosylated pentaglycosylceramide (Fig. 5:II) could also be seen at *m/z* 1359.8, although other structures such as globopentaosylceramide (n16:0) would give the same signal. Oxonium ions consistent with a fucosylated pentasaccharide (Fig. 5:II) were seen at *m/z* 189.1 and 828.5. Smaller peaks (not indicated) consistent with immonium ions for a hexaglycosylceramide (Fig 5:III) were seen at *m/z* 1591.0 (n16:0), 1619.1 (n18:0) and *m/z* 1703.2 (n24:0). There was also evidence of immonium ions for a fucosylated heptaglycosylceramide (not indicated) at *m/z* 1765.2 (n16:0) and with smaller peaks up to *m/z* 1877.3 (n24:0).

In the permethylated spectrum, oxonium ions (Fig. 4B) for a fucosylated pentasaccharide were seen at *m/z* 189.1, 842.3 and 638.2, for a difucosylated hexasaccharide at *m/z* 189.1, 812.3, 1016.3 and a hexaglycosylceramide at *m/z* 464.1, 913.3, and 1117.4 (Fig. 5). The signals at *m/z* 606.3 (638.2–32), and *m/z* 1087.4 (1117.4–32) represent fragments which have lost methanol. The signal at *m/z* 401.9 represents an internal HexNAc-dHex fragment (812-393-17) diagnostic of a type 1 chain [24]. Signals at *m/z* 464.1 (and 432.0 less methanol) were indicative of Hex-HexNAc and/or HexNAc-Hex terminal structures. The presence of terminal HexNAc (possibly from residual globoside) was seen at *m/z* 259.9.

LSIMS produced molecular ions [M + 1] (Fig. 6) for a difucosylated hexaglycosylceramide with a range of different ceramides at *m/z* 1790.2 (tl8:0-n16:0), 1820.2 (tl8:0-h16:0), 1874.3 (tl8:0-n22:0), 1904.4 (tl8:0-h22:0) and 1932.3 (tl8:0-

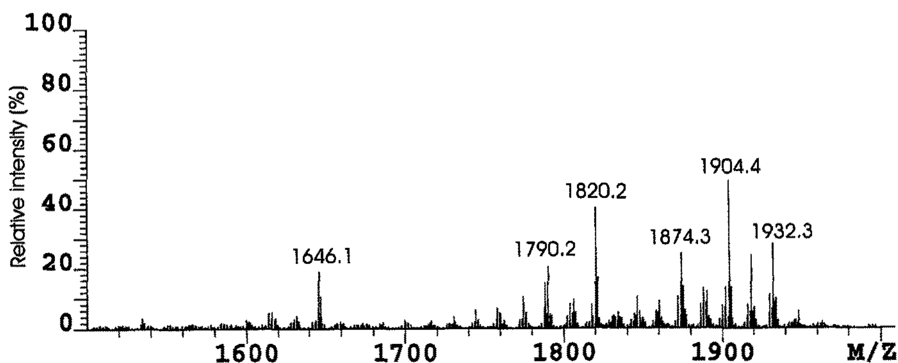

Figure 6. LSIMS spectrum of permethylated-reduced glycolipids showing molecular ions for a difucosylated hexaglycosylceramide (Le^{b}) as isolated in fraction 7.

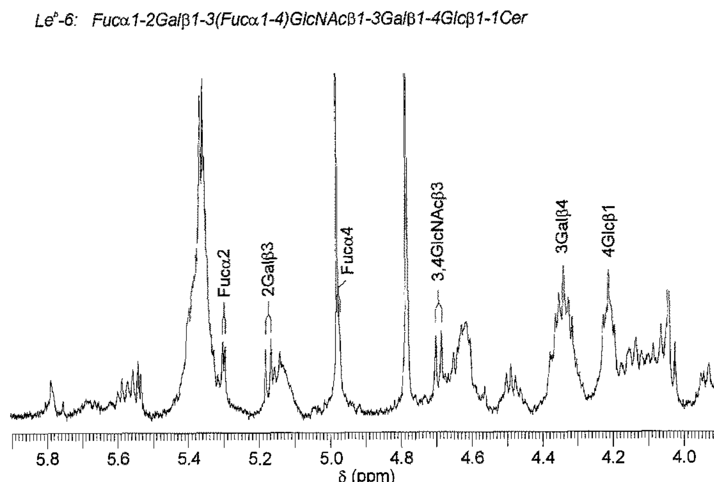

Figure 7. Anomeric region of the 500 MHz proton NMR spectrum of the permethylated-reduced glycolipids of fraction 7, recorded at 40°C in $CDCl_3$. The Le^b-6 signals are indicated in the spectrum and its carbohydrate structure is given on top. Three large impurities are seen at 4.78 ppm, 4.98 ppm, and around 5.36 ppm.

h24:0). Although the large peak seen at *m/z* 1646.1 correlated with an immonium ion from a difucosylated hexaglycosylceramide, it is more likely to have arisen from molecular ions (1645.2 + 1) of either a monofucosylated pentaglycosylceramide (t18:0-h16:0) or globopentaosylceramide (t18:0-n16:0).

All of this small fraction was permethylated and reduced for MS analysis, but retrospectively 500 MHz NMR was attempted. One of the difficulties with NMR of a permethylated-reduced sample is that interpretation is largely limited to the anomeric region of the spectrum. All the same, despite the complex appearance with several clusters of unresolved signals, a set of well resolved doublets from the dominating Le^b glycolipid was present.

In the anomeric region (Fig. 7) it was possible after resolution enhancement (not shown), to distinguish two α-doublets, one at 5.297 ppm and the other at 4.973 ppm (overlapping a large impurity singlet). They could be identified as arising from an α1,2 linked fucose and an α1,4 linked fucose respectively. Also clearly identifiable were two β-doublets at 5.172 ppm and 4.690 ppm that could be assigned to a 2-substituted β1,3 linked galactose and a 3,4-substituted β1,3 linked *N*-acetamido glucose, respectively. All these signals agreed with published data from this laboratory [25]. The anomeric signals from the internal lactosyl unit were present at the expected positions (≈ 4.3 ppm for Galβ and ≈ 4.2 for Glcβ), but due to overlap their exact chemical shifts could not be measured. The same was true for the C-5 protons of the two fucoses, appearing in clusters at ≈ 4.60 ppm and ≈ 4.45. These signals identified the presence of a Le^b-6 glycolipid in this fraction. No other structures could be conclusively identified in the NMR spectrum. A signal at 5.785 ppm could be assigned to a Fucα1-3 anomeric proton [25], though its small coupling (≤2.5 Hz) was not fully resolved. This Fucα1-3 could belong to either Le^x or Le^y. Other signals at chemical shifts corresponding to previous assignments made for Le^x and Le^y were present but intensities were (as far as they could be estimated) often lower than their fucose signal. Together with the MS-data above the presence of both Le^x and Le^y seemed very likely. On the other hand, the identification of H type 1 and H type 2 was made impossible by an impurity (around 5.36 ppm) covering the area of their fucose anomeric signals. Since this large signal (signals) is not matched in intensity by any other anomeric signals, and its apparent spin-spin coupling of 4.5 Hz was larger than one would expect for an α-anomeric signal, it must belong to non-glycosphingolipid impurities.

Evidence for Le^b-6 in this fraction was compelling. By TLC, a glycolipid with the mobility of a hexaglycosylceramide was found to immunostain with two different anti-Le^b reagents. By MS analysis, sequence and molecular ions for a fucosylated hexaglycosylceramide were conclusively identified. By NMR analysis, signals diagnostic for Le^b-6 were found. These data, taken together, were conclusive for the identification of Le^b-6. In addition, fraction 7 also contained a fucosylated pentasaccharide, probably Le^x (as in fraction 6), together with some evidence of Le^y, a hexaglycosylceramide and a fucosylated heptaglycosylceramide. Trace amounts of globopentaosylceramide may also have been present.

Fraction 8 glycolipids migrated by TLC from approximately hepta- to nonaglycosylceramides (Fig. 2). This fraction was reactive with anti-Le^a in the nonaglycosylceramide region. Trace reactivity was also seen in this region with anti-Le^b but not with anti-Le^bH, suggesting the presence of an extended Le^b structure. Reactivity was seen with anti-Le^c in the nonaglycosylceramide region and additionally in the heptaglycosylceramide region.

EI-MS analysis of this fraction found multiple compounds which were not possible to fully resolve (not shown). Partial separation and tentative identification of some structures was achieved by controlled temperature distillation and by selective ion monitoring. In the permethylated-reduced sample, immonium ions were found at *m/z* 1765, consistent with a n16:0 fucosylated heptaglycosylceramide, while the peak found at *m/z* 1939 probably represents this same linear structure, but with a second dHex unit, i.e. a difucosylated octaglycosylceramide (1765 + 174). Immonium ions consistent with a n16:0 monofucosylated branched nonasaccharide structure were found at *m/z* 2200–2201. EI-MS of the permethylated sample produced sequence specific fragments consistent with the above structures.

The permethylated-reduced NMR spectrum of this very small fraction was not possible to interpret in terms of structures present. The mixture of relatively large glycolipids gave rise to clusters of overlapping signals at expected chemical shifts. There were also several peaks from impurities that overshadow some glycolipid signals. Compared with the previous fraction, all the Le^b signals had disappeared.

Discussion

Inappropriate immunological expression of Lewis antigens is often seen in different tissues and fluids [2–9]. The detection and characterization of Lewis antigens by immunological techniques is however by no means unequivocal, as chemically related structures may crossreact. Monoclonal and polyclonal anti-carbohydrate antibodies always have some degree of crossreactivity [13]. Furthermore, samples of biological origin may have greater diversity than the range of synthetic structures against which an antibody is known to react. Thus, unexpected immunological findings must be supported by structural studies.

This paper describes an investigation of the non-acid glycolipids in the plasma of a single Le(a-b-) secretor individual, by immunostaining with highly characterized antibodies, and identification of structures by EI-MS, LSIMS and proton NMR spectroscopy. The plasma glycolipids of this Lewis-negative secretor-positive individual had previously been reported, on the basis of immunoreactivity, to have Le^b-6 glycolipids [3]. We now confirm, in this individual, the identity of these anti-Le^b reactive glycolipids as Le^b-6. The glycolipid immunological profile of the sample studied here is identical to that of several other individuals of the same phenotype [3], and so it is reasonable to assume that this result is representative of Lewis-negative secretors. Similarly, Le^a antigens can be expected to be present in Lewis-negative non-secretor plasma [3] and have been structurally identified in small intestinal glycolipids from a Lewis-negative non-secretor individual (S. Henry, unpublished observation).

Previously several blood group related glycolipids have been structurally determined in the plasma of Lewis-negative samples [26–29], but the structural identification of Le^b in a Lewis-negative individual has not been previously described. This glycolipid is usually only found in Lewis-positive secretor-positive individuals [24, 27, 30]. This discrepancy could be due to enhanced sensitivity of techniques more than a decade later, and a fractionation strategy based on glycolipid immunoreactivity. About 0.5% of the total neutral glycolipid extract of the Le(a-b-) sample was Le^b, which is about 20 times less than that usually found in a Le(a-b+) sample.

Although it cannot as yet be demonstrated which transferase is responsible for the formation of Lewis antigens in Lewis-negative samples, it appears that Le(a-b-) individuals may have some α-4-fucosyltransferase activity. A low level of α-4-fucosyltransferase activity has been found in Lewis negative individuals (A. Chester, personal communication) and in normal bladder urothelium [31]. It is possible that some, or all, of the Lewis negative alleles, which show considerable genotypic heterogeneity [32–35], may possess limited α-4-fucosyltransferase activity. Alternatively, a different, non-Lewis α-4-fucosyltransferase may be involved. A good candidate is FUT5 [36, 37], which has recently been found to work as well on type 1 as on type 2 precursors (R. Mollicone, unpublished observation).

In addition to the detection of Le^b, structural analysis of this Le(a-b-) secretor sample revealed many known structures. As expected, the dominating blood group glycolipid was H-5-1, which is consistent with the accepted biosynthetic pathway for Lewis antigens, where lactotetraosylceramide (type 1 precursor, or Le^c) is fucosylated by the secretor transferase to form H-5-1 (Le^d). It can be shown here that this conversion is complete (or at least almost complete) because no lactotetraosylceramide was detectable in this Le(a-b-) secretor. The NMR spectrum shown here of H type 1 differs slightly from published data [22], but is identical to reference H type 1 at Göteborg University.

In agreement with published reports, mono-, di-, tri-glycosylceramide, globoside, paragloboside, Le^X-5, Le^Y-6 were seen in the plasma of this Le(a-b-) secretor sample [27, 29, 38–40]. Traces of globopentaosylceramide were also found. In contrast to a previous report [29] H type 2 was not detected, nor was it found by immunochemical means using various anti-H type 2 reagents (results not shown).

EI-MS evidence of three known extended structures; a linear hexaglycosylceramide, a linear monofucosylated heptaglycosylceramide and a monofucosylated branched nonasaccharide were seen as described in pooled group O Le(a-b-) plasma by Hanfland [26]. Fraction eight contained these and other polyglycosylceramides, but unfortunately, due to their very low concentrations and complexity, structures could not be fully determined.

Results in this paper confirm that the aberrant immunological detection of Lewis antigens in Lewis negative individuals is probably due to the detection of trace amounts of Lewis antigens made by individuals of this phenotype.

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